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2-Mercaptoethanol and *n*-acetylcysteine enhance T cell colony formation in AIDS and ARC

J. WU, E. M. LEVY & P. H. BLACK *Department of Microbiology, Boston University School of Medicine, Boston, MA*

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SUMMARY

One contributing factor to the loss of T cells in AIDS may be the impaired ability of T cell precursors to expand, as reflected in a decreased ability of patient cells to form T cell colonies in agar. We and others have noted such a defect in people with AIDS and ARC, and have found that suppressor cells and suppressive plasma contribute to decreased T-CFC formation. We report here that the reducing agents 2-mercaptoethanol (2-ME) and *n*-acetyl cysteine (NAC) can enhance colony formation *in vitro*. In part, 2-ME can reverse the defect in T cell colony-forming cells (T-CFC) formation by overcoming the effect of suppressor cells. In a group of 46 AIDS patients, T-CFC formation was initially $42 \pm 8\%$ (mean $\pm$ s.e.) that of control levels. 2-ME caused an increase of $401 \pm 76\%$ in T-CFC formation which was significantly greater than the increase in control T-CFC formation; it also significantly enhanced T-CFC formation by cells from ARC patients. Suppressor cell activity from ten AIDS patients decreased from $58 \pm 21\%$ to $12 \pm 10\%$ when 2-ME was added. Similar data were obtained from 14 ARC patients. NAC, a related antioxidant with low toxicity, also enhanced T-CFC in cells of AIDS and ARC patients. Vitamin C generally did not increase T-CFC formation. The data suggest that certain antioxidants such as 2-ME and NAC may be useful in treatment protocols to enhance T cell numbers in patients with AIDS or ARC.

Keywords AIDS T cell colony-forming cells 2-mercaptoethanol *n*-acetylcysteine

INTRODUCTION

A spectrum of immunological defects has been documented in patients with symptomatic human immunodeficiency virus (HIV) infections, including a profound defect in the ability of mononuclear cells from patients with AIDS and ARC to form T-cell colonies in a semi-solid medium (Fauci *et al.*, 1984; Winkelstein *et al.*, 1985, 1988; Levy *et al.*, 1986). The decreased ability to form colonies is likely to be a marker of a decreased ability to replace cells destroyed by HIV, and therefore could strongly contribute to the pathogenesis of HIV. Mercaptoethanol (2-ME) has been shown to enhance various immunological *in vitro* reactions, e.g. lymphocyte transformation. More specifically, it has been reported that 2-ME can overcome the effect of a suppressor factor produced by HIV infected cells (Laurence, Gottlieb & Kunkel, 1983). We used 2-ME and several other antioxidants to determine if they would enhance T-cell colony formation by patient cells. We report here an effect of 2-ME and the related compound *n*-acetylcysteine (NAC) on T cell colony-forming cells (T-CFC) of AIDS and ARC patient cells. Like 2-ME, NAC has sulphydryl groups which are important to its activity. Overall, Vitamin C—a structurally unrelated antioxidant— had no effect on colony formation. Although 2-ME was more effective than NAC, 2-ME cannot be used clinically because of its toxicity, unlike NAC. NAC should be considered as a possible adjunctive treatment in AIDS and ARC.

Correspondence: Dr Elinor Levy, Department of Microbiology, Boston University School of Medicine, 80 East Concord Street, Boston, MA 02118, USA.

MATERIALS AND METHODS

The study included 54 AIDS patients who had had a diagnosis of *Pneumocystis carinii* pneumonia (PCP) or Kaposi's sarcoma, and 73 patients with constitutional symptoms of HIV infection (referred to hereafter as ARC patients for simplicity). Patients were recruited from the Immunodeficiency Clinic at Boston City Hospital to participate in this study. Fifty-three healthy volunteers served as controls.

Isolation of mononuclear cells

Heparinized blood was centrifuged at 400 ***g*** for 10 min. Plasma was aspirated off. The packed cells were diluted to twice the original blood volume with Ca^{2+} and Mg^{2+}-free Hanks' balanced salt solution (HBSS). The diluted blood was underlayered with Isolymph (Gallard-Schlesinger, Carle Place, NY) and centrifuged for 30 minutes at 400 ***g***. Cells were collected from the interface, washed three times with HBSS, and resuspended in McCoy's 5a medium (GIBCO, Grand Island, NY).

Table 1. Effect of 2-ME in T-CFC formation in AIDS and ARC

	Mean ± s.e.	+2-ME	P*	Percentage increase†
Control ($n=26$)	357 ± 41	524 ± 53	<0·001	168 ± 16%
AIDS ($n=46$)	104 ± 16	187 ± 27	<0·001	401 ± 76%
Control ($n=30$)	346 ± 39	509 ± 56	<0·001	178 ± 16%
ARC ($n=65$)	149 ± 14	273 ± 23	<0·001	400 ± 85%

* Paired *t*-test of T-CFC with vs without 2-ME.
† See Results.

T cell colony assay

The T-CFC assay was performed as previously described (Levy *et al.*, 1986). Briefly, a two-layer agar system was used. Cells were grown in 24-well culture plates. The underlayer consisted of phytohaemagglutinin (PHA) (1%) (Difco), 10% (v/v) human T cell polyclone, a supernatant containing interleukin-2 (IL-2) (Collaborative Research, Bedford, MA), 15% fresh human plasma from a healthy control subject unless indicated otherwise, and 0·5% agar. Where indicated, 2-ME, NAC, or Vitamin C were added to the underlayer. Cells were added in a thin overlayer consisting of 2×10^4 cells in McCoy's 5A medium with 15% human plasma and 0·3% agar. Cultures were incubated in a 5% CO_2 atmosphere at 37°C with a relative humidity of 85% in a Napco 7300 incubator for 7 days. Only tight clusters containing a minimum of 20 cells were counted as colonies. In some experiments, patient and control cells were admixed in the upper layer. In these experiments the number of expected colonies was calculated from the sum of colonies observed when patient and control cells were grown independently. The percentage suppression was calculated

$$\left(1-\frac{\text{T-CFC observed}}{\text{T-CFC expected}}\right) \times 100.$$

The relative growth caused by the patient plasma was calculated from the number of colonies formed when control cells were grown in the presence of patient plasma, divided by the number of colonies which grew from control cells in control plasma multiplied by 100.

RESULTS

Effect of 2-ME on colony formation in agar

Peripheral blood mononuclear cells (PBMC) were evaluated for their ability to form T-cell colonies in agar. PBMC from patients with AIDS and ARC were generally depressed in this colony-forming ability. Cells from the AIDS patients formed 104 ± 16 colonies/2×10^4 PBMC (mean ± s.e.; $n=46$) compared with 357 ± 41 colonies from cells obtained from controls ($n=26$) ($P<0·001$) (Table 1). Thus, as previously reported, patients with AIDS were impaired in their ability to form T-CFC (Levy *et al.*, 1986). The addition of 2-ME at concentrations of 5×10^{-5} M stimulated colonies of both control subjects and AIDS patients. In the presence of 2-ME, as shown in Table 1, the number of colonies of control cells was increased to 524 ± 53 ($P<0·001$). In comparison, the mean response in the patients with AIDS was increased to 187 ± 27 ($P<0·001$). Expressed as a relative effect, 2-ME caused an increase of 401 ± 76% in AIDS patient T-CFC formation, compared with an increase of 168 ± 16% for their controls. The percentage increase was calculated thus:

$$\frac{\text{No. of T-CFC with 2-ME}}{\text{No. of T-CFC without 2-ME}} \times 100.$$

This may appear larger than the mean of T-CFC with and without 2-ME would suggest. As an example of how this occurs, if we had 2 subjects whose initial T-CFC values were 10 and 100, respectively, and whose values were 100 and 200, respectively, in the presence of 2-ME, the average T-CFC value would have increased from 55 to 150, but the percentage increase would be 600%, i.e. (1000 + 200)/2. The differences between the degree of enhancement of T-CFC for AIDS patients and their controls were significant ($P<0·001$). Qualitatively, the size of the colonies was also frequently increased in the presence of 2-ME. This indicates that 2-ME can significantly improve the ability of AIDS patient cells to form T-CFC, and does so preferentially compared with controls.

Similar data were obtained with cells from ARC patients. Cells from ARC patients formed 149 ± 14 colonies/2×10^4 PBM cells ($n=65$), compared with 346 ± 39 colonies from cells obtained from controls ($n=30$) ($P<0·001$). In the presence of 2-ME, as shown in Table 1, the number of colonies from control cells was increased to 509 ± 56. In comparison, the mean response in patients with ARC was increased to 273 ± 23. The difference between T-CFC formation in the presence and absence of 2-ME is highly significant for both the ARC group and for the control group. In the presence of 2-ME, the number of control T cell colonies was increased by 178 ± 16%. The number of T cell colonies in ARC patients was increased by 400 ± 85%. The relative effect of 2-ME on patients' cells was again significantly greater than on control cells ($P<0·001$).

Effect of 2-ME on suppressor cell activity

Since 2-ME had been demonstrated to inactivate a suppressor factor produced by HIV-infected cells (Laurence, Gottlieb & Kunkel, 1983), we investigated the possibility that 2-ME would decrease suppressor cell activity. The patient cells were mixed 1:1 with control cells and the degree of suppression calculated from the observed/expected value. Suppression >25% was observed for 14 ARC and ten AIDS patients. Considering only those experiments in which suppressor cell activity of >25% was demonstrated, the percentage of suppressor cell activity was 47 ± 6% for AIDS patients. The suppressor cell activity decreased to 12 ± 10% when 2-ME was added ($P<0·02$). In ARC patients, the percentage of suppressor cell activity was 54 ± 4%. This decreased to 8 ± 14% when 2-ME was added ($P<0·01$). Thus, 2-ME was effective in reducing suppressor cell activity.

Effect of 2-ME on suppression by patient plasma

AIDS and some ARC patient plasma can significantly suppress control T-cell colony formation (Levy *et al.*, 1986). 2-ME was added to control cells with patient plasma to determine whether 2-ME would reduce the suppression of patient plasma on control T-CFC. Although there was an increase from 173 ± 21 to 372 ± 40 colonies/2×10^4 cells when 2-ME was added to control cells in patient plasma, a similar enhancement was

Table 2. NAC effect on T-CFC formation

	Mean+s.e.	+NAC	P*	Percentage increase†
Control ($n=22$)	349±53	549±16	<0·001	186±22%
ARC ($n=23$)	153±32	243±45	<0·001	205±30%
AIDS ($n=14$)	116±17	213±40	<0·05	234±53%

* Paired t test of T-CFC with and without NAC.
† See Results.

Table 3. Effect of Vitamin C on T-CFC formation in AIDS and ARC

	Mean±s.e.	+Vitamin C
Control ($n=18$)	323±46	310±48
Patient ($n=35$)	142±23	143±27

observed when 2-ME was added to the same cells in control plasma. The percentage of suppression of control cells by plasma from AIDS or ARC patients was 52±10% before adding 2-ME. In the presence of 2-ME, despite an increase in plating efficiency, the percentage of suppression by patient plasma remained at 48±8%.

Effect of NAC on colony formation in agar

Although 2-ME has dramatic effects on colony formation *in vitro*, due to its toxicity it is not a compound that can be used clinically. There are, however, related compounds which can be considered for clinical use to enhance clonal expansion of T cells. One such compound is NAC. Like 2-ME, NAC possesses a free sulphydryl group that can reduce disulphide bonds. We observed that NAC also has the effect of enhancing T-CFC formation with cells from AIDS and ARC patients. Cells from the ARC patients formed 153±32 colonies/2×10^4 cells (mean±s.e.; $n=23$), compared with 349±53 colonies from cells obtained from controls ($n=22$) ($P<0·001$) (Table 2). NAC at concentrations of 10^{-5} M stimulated both control cells and ARC patient cells. Colonies of control cells were increased to 549±16 ($P<0·0001$). In comparison, the mean response in the 23 patients with ARC was increased to 243±45 ($P<0·001$). The difference between T-CFC formation in the presence and absence of NAC is also significant for the AIDS group using a paired t test ($P<0·05$). NAC causes an increase of 205±30% in T-CFC formation by ARC patients cells and 234±53% in AIDS patient cells. This indicates that NAC can significantly improve the ability of AIDS and ARC patient cells to form colonies—although the improvement was equally high in patients and controls. NAC at concentrations of $1–2\times10^{-5}$ M was found to be optimal for stimulation of control and patient T cell colony formation. NAC at a concentration $>10^{-3}$ M inhibited the T-CFC formation. In contrast to the marked stimulation of T-CFC formation, neither 2-ME nor NAC enhanced the mitogen response of patient cells to PHA in bulk culture, suggesting an effect that was somewhat specific for T-CFC formation (data not shown).

Vitamin C, another anti-oxidant which has been used in uncontrolled trials of patients with AIDS, generally did not increase T-CFC formation (Table 3). Thus, not all anti-oxidants increase T-CFC formation.

DISCUSSION

It has been shown that 2-ME enhances various immunological responses *in vitro* (Axelsson *et al.*, 1976; Metcalf, 1976; Donald & Claudette 1978; Claesson, Flad & Opitz, 1979; Hoffeld & Oppenheim, 1980). 2-ME is also generally known to support cell survival and function *in vitro*. Additionally, it had been reported that 2-ME can overcome a soluble suppressor factor made by cells from AIDS and ARC patients (Laurence, Gottlieb & Kunkel, 1983). We therefore postulated that 2-ME might help restore the ability of patient cells to form T-CFC. When 2-ME (5×10^{-5} M) was added to cultures with AIDS and ARC patient cells, it significantly and preferentially increased patient T-CFC formation. It was noted that 2-ME reduced patient suppressor cell activity; in mixing experiments, 2-ME greatly decreased the suppression of AIDS and ARC patient cells on control T-CFC formation. There was, however, no differential effect in the enhancement of control T-CFC formation by patient compared with control plasma. These data suggest that the mechanism by which 2-ME enhances T-cell colony formation is partly related to its anti-suppressor cell activity (Laurence, Gottlieb & Kunkel, 1983; Laurence & Mayer 1984).

To investigate the possibility that other anti-oxidants with possible clinical relevance would enhance T cell colony formation in AIDS and ARC patients, NAC and Vitamin C were studied. NAC possesses free sulphydryl groups like 2-ME. Like 2-ME, NAC can significantly improve the ability of AIDS and ARC patient cells to form T-cell colonies. Vitamin C, however, was not effective in this assay.

The mechanism by which 2-ME and NAC enhances T-cell formation may involve reduction of cellular components that are essential for mitosis. 2-ME and NAC both have sulphydryl groups which are required for the mitogenic and adjuvant effects of these compounds. Evidence that lymphocyte triggering involved sulphydryl interactions was gathered by Chaplin & Wedner (1978), who found that lectin-dependent lymphocyte activation was inhibited by a variety of sulphydryl-combining reagents.

It is likely that 2-ME and NAC work in part through their action on glutathione (GSH) levels. Fanger *et al.* (1970) showed that high concentrations of L-cysteine and GSH enhanced the responses of human and rabbit peripheral blood lymphocytes to mitogens. Additionally, Heber-Katz & Click (1972) showed that relative to L-cysteine and GSH, much lower concentrations of 2-ME were needed for enhancement of the mixed lymphocyte reaction. NAC is a precursor of intracellular GSH. NAC treatment generally increases intracellular GSH and restores its levels following GSH depletion (DeFlora, Rossi & DeFlora, 1986). GSH can be either spontaneously or enzymatically oxidized to the disulphide form GSSG under culture conditions.

In this form, GSSG is a potent inhibitor of protein synthesis (Hoffeld & Oppenheim, 1980). The inhibition by GSSG can be reversed by NAC and 2-ME (DeFlora, Rossi & DeFlora, 1986). Cysteine's *n*-acetyl derivative has been found to be very effective in protecting cells from reactive oxygen species and other classes of toxic components (Moldeus, Cotgreave & Berggren, 1986; Yunis, Lim & Akimura, 1986; Ziment, 1986). Thus, 2-ME and NAC may improve the oxidation state of cellular components that are essential to T cell formation in AIDS and ARC and also protect cells from the by-products of inflammatory reactions and possibly against some of the toxic side effects of other therapeutic drugs. It is interesting to note that the ancient Chinese used burned hair as a drug; its effects might have been related to the L-cysteine content of the hair (Ziment, 1986). In our experiments, the effect of 2-ME and NAC were selective for T-CFC formation. There was no effect on mitogen stimulation. This could allow an increase in T-cell replacement without enhancing immune activation, which is associated with enhanced HIV replication.

Our data suggest that anti-oxidants containing sulphydryl groups such as 2-ME and NAC may be useful in treatment protocols to enhance T-cell numbers in patients with AIDS or ARC. 2-ME cannot be used clinically because of its toxicity. NAC, although somewhat less effective, is an acceptable and safe clinical agent that is already used for topical administration in the lungs as a mucolytic medication and systemically for the management of acetaminophen poisoning and the scavenging of free radicals liberated by cancer chemotherapy drugs. Thus, NAC may be compared with many agents that have multiple actions (Ziment, 1986). Treatment can be given intravenously, or in large doses orally, and the doses of NAC required are generally safe. The usual oral clinical dose of NAC is 200 mg/kg body weight. The concentration which was active *in vitro* should be easily achievable *in vivo* (Moldeus, Cotgreave & Berggren, 1986).

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